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Live, attenuated strains of Bacillus anthracis lacking either the capsule plasmid pX02, the toxin plasmid pX01, or both plasmids, were tested for their efficacy as vaccines against intravenous anthrax toxin challenge in Fischer 344 rats and against aerosol or intramuscular, virulent anthrax spore challenge in Hartley guinea pigs. Only those animals immunized with toxigenic, non-encapsulated (pX01+, pX02-) strains survived toxin or spore challenge and demonstrated post-immunization antibody titers to the three components of anthrax (protective antigen, lethal factor, and edema factor). Immunization with two

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non-toxigenic, encapsulated (pXO1-, pXO2+), Pasteur vaccine strains neither provided protection nor elicited titers to any of the toxin components. Therefore to successfully immunize against anthrax toxin or spore challenge, attenuated, live strains of B. anthracis must produce toxin components specified by the pXO1 plasmid.

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Immunization Studies With Attenuated Strains of *Bacillus anthracis*

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

Live, attenuated strains of Bacillus anthracis lacking either the capsule plasmid pX02, the toxin plasmid pX01, or both plasmids, were tested for their efficacy as vaccines against intravenous anthrax toxin challenge in Fischer 344 rats and against aerosol or intramuscular, virulent anthrax spore challenge in Hartley guinea pigs. Only those animals immunized with toxigenic, non-encapsulated (pX01(+), pX02(-)) strains survived toxin or spore challenge and demonstrated post-immunization antibody titers to the three components of anthrax toxin (protective antigen, lethal factor, and edema factor). Immunization with two non-toxigenic, encapsulated (pX01(-), pX02(+)), Pasteur vaccine strains neither provided protection nor elicited titers to any of the toxin components. Therefore to successfully immunize against anthrax toxin or spore challenge, attenuated, live strains of B. anthracis must produce toxin components specified by the pX01 plasmid.

INTRODUCTION

Virulent strains of Bacillus anthracis possess two plasmid-mediated virulence factors (21): a poly-D-glutamic acid capsule (11), and a tripartite exotoxin (1, 23), consisting of protective antigen (PA), edema factor (EF), and lethal factor (LF). Production of the toxin is controlled by the 110-megadalton (MDa) plasmid pXO1 (formerly pBA1) (16), while synthesis of the capsule is dependent upon the 60-MDa plasmid pXO2 (7, 26). Thus, fully virulent strains ($pXO1^+$, $pXO2^+$) produce both toxin and capsule; $pXO1^+$, $pXO2^-$ strains produce toxin only; $pXO1^-$, $pXO2^+$ strains produce capsule only; $pXO1^-$, $pXO2^-$ strains produce neither toxin nor capsule (7).

In the United States, the currently licensed human vaccine against anthrax consists of an aluminum hydroxide precipitate of supernatant material from a toxigenic non-encapsulated strain of B. anthracis, V770-NP1-R (18). Immunization with this vaccine, which is composed primarily of protective antigen (9), requires a series of six doses over an 18-month period, followed by boosters at yearly intervals. For immunization of livestock against anthrax, a suspension of viable spores of the toxigenic, non-encapsulated Sterne strain is commonly employed (25).

The first anthrax vaccines which were widely used in livestock during the late 1800s and early 1900s were the Pasteur-type vaccines (17, 28, 29), which consisted of cultures of virulent anthrax bacilli attenuated by growth at 42-43°C. Unfortunately, these vaccines varied greatly in their degrees of attenuation (28, 29). Some of the cultures were sufficiently virulent to kill the animals being inoculated (13), while others were so attenuated that they conferred no immunity to infection (27, 28). An explanation for the molecular

mechanism of attenuation of Pasteur-type vaccines was recently proposed by Mikesell et al., who demonstrated that culturing B. anthracis at 42.5°C cures the bacillus of the toxin plasmid pXO1 (16), and thus converts fully virulent $pXO1^+$, $pXO2^+$ cells to avirulent $pXO1^-$, $pXO2^+$ cells.

The studies reported here are part of the continuing research toward the development of a more protective and less reactogenic human anthrax vaccine. Their purpose was to compare B. anthracis strains lacking one or both primary virulence factors (and their respective plasmids) with respect to their efficacy in immunizing experimental animals against anthrax toxin or spore challenge, and to compare the effectiveness of various vaccine administration regimens. Additionally, these studies sought to further elucidate the nature of Pasteur's early anthrax vaccines and to provide an explanation for their immunizing efficacy.

MATERIALS AND METHODS

Bacterial strains. The B. anthracis strains used in this study are listed in Table 1.

Experimental animals. Male, 200-300g, Fischer 344 rats were employed in the toxin challenge studies. Female, 275-325g, Hartley guinea pigs were used for the spore challenge studies.

Immunization and challenge studies. Fischer 344 rats (5 per group) and Hartley guinea pigs (7-9 per group) were immunized intramuscularly (i.m.) either with attenuated-strain vegetative cells from late log-phase R medium cultures (19), or with the currently licensed human anthrax vaccine (2, 18) prepared by the Michigan Department of Public Health and designated in this report as MDPH. For anthrax toxin challenge studies, groups of rats were immunized with 2 x 10^7 CFU of B. anthracis Sterne, Pasteur 4229, ΔVollum 1B-1, ΔVollum 1B-3, ΔSterne-1, or ΔV770-NP1-R-strain cells twice weekly for four weeks, then given a final boost at 5 1/2 weeks. At six weeks, one ml of R medium culture supernatant containing 62 toxic units (TU) (8) of crude Vollum 1B anthrax lethal toxin (approximately 25 μg PA and 3 μg LF) (6) was injected into the dorsal penile veins of the rats, and times to death (TTD) in min were recorded. For i.m. spore challenge studies, several vaccination regimes employing various B. anthracis strains were employed. Each group of guinea pigs was immunized according to one of the following schedules: i) two injections, spaced two weeks apart, of 10^6 CFU B. anthracis Sterne-strain cells; ii) two injections, spaced two weeks apart, of 10^7 CFU B. anthracis ΔVollum 1B-1, Pasteur 4229, or ΔSterne-1-strain cells; iii) four biweekly

injections of 10^8 CFU B. anthracis ΔTexas-1 or ΔTexas-2-strain cells; and iv) four biweekly injections of B. anthracis Texas-strain cells with the first injection containing 500 CFU; second, 1500 CFU; third, 5000 CFU; and fourth, 10,000 CFU. Two weeks following the final immunization, all animals received 1500 (approximately 30 i.m. LD_{50}) Vollum 1B spores. Times to death in days were recorded. and Harmonic mean TTD were calculated in order to provide a better indication of the overall survival of each group of guinea pigs (3, 4). For aerosol spore challenge studies, groups of guinea pigs were immunized either with B. anthracis Sterne, ΔVollum 1B-3, ΔTexas-1, ΔSterne-1, Pasteur 4229, or Pasteur 6602-strain cells in four biweekly doses of 10^7 CFU, or with MDPH in three biweekly doses of 0.5 ml. Two weeks following the final immunization, the guinea pigs were placed in a modified Henderson aerosol apparatus (12, 15, 20) for 10 minutes and allowed to inhale either 1.58×10^6 (26 aerosol LD_{50}) or 2.09×10^6 (35 aerosol LD_{50}) virulent Vollum 1B spores. Times to death in days were recorded, and the Harmonic mean TTD were calculated.

Serological studies. PA, EF, and LF were purified from R medium culture supernatants of B. anthracis Sterne as previously described (14). Post-immunization sera from rats and guinea pigs were titered for antibody against the three toxin components by enzyme-linked immunosorbent assay (ELISA). For the assays, microtiter plate (Linbro) wells were coated with PA, EF or LF (100 µl of a 1 µg/ml solution in 0.05 M sodium borate, pH 9.5) Following incubation for 4 h at 37°C or overnight at 4°C, 160 µl of either bovine serum albumin (BSA) or gelatin (1 mg/ml in 5 mM HEPES, 0.15 M NaCl, pH 7.5) was added to each well, and the plates were reincubated for 30 minutes at 25°C. The wells were washed twice with 300 µl phophate buffered saline + Tween 20

(PBST) (0.85% sodium chloride, 10 mM sodium phosphate, 0.05% Tween 20). One hundred and fifty microliters of PBST + 5% fetal calf serum (PBSTF) or 0.1% gelatin (PBSTG) was added to each well. Fifty microliters of each serum sample to be tested was added to the first well in a row, then serially diluted 1:4 down the row. The plates were incubated overnight at 4°C and then washed twice with PBST. For guinea pig sera, each well received 100 µl of horseradish-peroxidase-Protein A conjugate in PBST (final conjugate concentration=1 µg/ml). For rat sera, each well received 100 µl of goat, anti-rat horseradish-peroxidase conjugate in PBSTG (final conjugate concentration=1 µg/ml). Plates were incubated at 25°C for 60 min then washed 5X with PBST. Substrate (2,2'-azino-bis(3-ethylbenzthiazdinesulfonic acid) (Sigma) was dissolved in 0.1 M sodium citrate, pH 4.0, to a concentration of 1 mg/ml. Hydrogen peroxide (0.003%) was then added, and the substrate + H_2O_2 solution was dispensed into the wells in 100 µl aliquots. After incubating 30 min, reactions were terminated by addition of 10% sodium dodecyl sulfate, 50 µl per well. Absorbances at 414 nm were determined with a Dynatech MR580 ELISA reader. Absorbance values which exceeded negative control values by greater than 2.1-fold were scored as positive (27). The mean titers of sera from immunized animals compared to those from unimmunized controls were statistically analyzed using one way analysis of variance and Fisher's Least Significant Difference Test (24).

To demonstrate that animals vaccinated with the various attenuated strains had received sufficient antigenic stimulus to engender an immune response, post-vaccination sera from the guinea pigs were examined for activity in a B. anthracis whole-cell ELISA system. Experimental conditions were identical to those described above, with two exceptions: a) 10^7 vegetative Sterne-strain cells in PBS were added to each well of a 96-well tissue culture plate (Costar), which was then dried overnight at 25oC and heated to 60oC for 30 min

to fix the cells to the wells; and b) rather than serial dilutions, a single 1:4 dilution of each serum sample was assayed. ELISA absorbance values for serum samples from vaccinated animals were qualitatively compared with values for sera from unvaccinated control animals. Statistical analyses on data were performed as described above.

RESULTS

Immunization of rats against toxin challenge. As demonstrated by the data in Table 2, rats immunized with the Sterne ($pXO1^+$, $pXO2^-$) strain cells were completely protected from lethal toxicity, and they exhibited significant titers to PA, LF, and EF ($P<0.05$). Rats immunized with strains containing only pXO2 or with strains containing neither plasmid, however, showed no evidence of protection in that they neither survived nor exhibited an extended TTD. Furthermore, no post-vaccination titers to PA, LF, or EF could be demonstrated in these animals.

Immunization of guinea pigs against intramuscular spore challenge. Since the toxigenic, nonencapsulated Sterne strain had been demonstrated to be an effective live vaccine against toxin challenge, it was decided in the i.m. spore challenge studies to also test another $pXO1^+$, $pXO2^-$ strain, Texas, and two of its plasmid-cured derivatives, ΔTexas-1 and ΔTexas-2. Texas is a clinical isolate of B. anthracis which, like the Sterne strain, produces all three toxin components but does not produce a capsule. Several different immunization regimens were employed to determine whether the size of dose or number of doses affected the guinea pig responses to spore challenge. The data in Table 3 demonstrate that immunization with Sterne-strain cells resulted in complete protection for the animals. Furthermore, immunization with Texas-strain cells was also completely protective. No animals vaccinated with B. anthracis cells lacking pXO1 survived challenge or had significant titers ($P<0.05$) to PA, LF, or EF. Texas-strain-immunized animals demonstrated titers to all three toxin components. Sterne-immunized guinea pigs had significant titers to PA and LF, but not EF.

Immunization of guinea pigs against aerosol spore challenge. Although vaccination with B. anthracis strains lacking pXO1 did not provide protection against intravenous toxin challenge or i.m. spore challenge, it remained possible that vaccination with such strains might afford protection against an aerosol spore challenge. The data in Table 4 demonstrate that although vaccination with either MDPH vaccine or live Sterne-strain cells afforded substantial protection to aerosol-challenged guinea pigs, immunization with strains lacking pXO1 neither afforded survival to the animals nor extended their mean TTD. Significant titers ($P<0.05$) to the three toxin components were evident only in sera from animals vaccinated with Sterne cells or MDPH.

Serological response to immunization. Post-vaccination sera from all groups of guinea pigs immunized with the various B. anthracis strains were compared in the whole-cell ELISA system with sera from an unimmunized control group. Vaccinated animals, regardless of the immunization regimen, demonstrated ELISA absorbance values significantly higher ($P<0.01$) than the absorbance values of the unvaccinated control group (data not shown). Thus, the immunization regimens for the various strains were sufficient in all cases to stimulate an antibody response to cell-surface antigens, albeit the response in animals receiving $pXO1^-$ strains was not protective.

DISCUSSION

From the data in this study, comparing the relative immunizing efficacies of ten attenuated strains of B. anthracis (see Table 1), it is clear that vaccination with cells lacking the pXO1 plasmid confers no protection against either anthrax toxin or spore challenge. This is in contrast to immunization with non-encapsulated, toxin-producing ($pXO1^+$, $pXO2^-$) strains such as Sterne or Texas, which are fully protective under a variety of immunizing regimens, i.e. several large doses, a few small doses, or a series of graded doses. The finding that ELISA titers were elicited to PA, LF, and EF only in animals vaccinated with strains containing plasmid pXO1 provides further evidence that functional toxin component structural genes are not present on the B. anthracis chromosome, but only on pXO1. Immunization with the MDPH human vaccine elicited titers to EF and LF as well as PA, indicating that the human "protective antigen" vaccine in fact contains all the toxin components. The protection and serological studies reported here thus indicate that to protect animals against a large anthrax toxin or spore challenge, one or more toxin components must be present in a chemical vaccine or produced by a live vaccine. The finding that vaccination with B. anthracis cells ($pXO1^-$, $pXO2^+$) producing capsule but not toxin was not protective supports previous reports that the D-glutamyl polypeptide capsule of the organism is not an important immunogen (10, 22). The finding, however, does not explain how Pasteur's heat attenuation regimen, which causes the selective loss of pXO1 (7, 16), was able to produce a successful live anthrax vaccine. It does further emphasize the apparent paradox between Pasteur's original vaccine strains and the Pasteur-type strains available from the American Type Culture Collection which are

completely ineffectual as immunogens. The current hypothesis to explain Louis Pasteur's mechanism of attenuation of B. anthracis is that by growing virulent cells at elevated temperatures, he induced the loss of the pXO1 toxin plasmid (16), resulting in an increased proportion of $pXO1^-$, $pXO2^+$ cells and a decreased proportion of virulent, $pXO1^+$, $pXO2^+$ cells in the cultures. Since our studies have clearly demonstrated that pure cultures of $pXO1^-$, $pXO2^+$ B. anthracis cells are not effective live vaccines, we believe the efficacy of Pasteur's vaccines can be attributed to the presence of small numbers of $pXO1^+$, $pXO2^+$ bacteria, and that subclinical infection by these bacteria would have induced a protective immune response. Evidence that Pasteur's vaccine did indeed contain virulent cells was provided by Pasteur himself. He demonstrated that serial passage of the attenuated cultures through day-old guinea pigs restored virulence (5), apparently by selecting for the virulent bacilli present. The fact that the Pasteur-type strains from the American Type Culture Collection contain only the pXO2 plasmid (7) while those from the Japanese collection contain both plasmids (26) must reflect the differing results obtained when pure culture technique was applied to Pasteur's mixed cultures without recognition that a mixture was present.

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TABLE 1. *B. anthracis* strains

Strain	Primary Virulence Factors	Plasmids[a]	Source
Vollum 1B	Toxin, Capsule	pXO1, pXO2	USAMRIID[b]
Sterne	Toxin	pXO1	USAMRIID
Texas	Toxin	pXO1	R.D. Welsh[c]
ΔVollum 1B-1	Capsule	pXO2	Derived from Vollum 1B Strain[d]
ΔVollum 1B-3	Capsule	pXO2	Derived from Vollum 1B Strain[d]
Pasteur 4229	Capsule	pXO2	ATCC[e]
Pasteur 6602	Capsule	pXO2	ATCC
ΔV770-NP1-R-1	None	None	Derived from USAMRIID Strain V770-NP1-R[d]
ΔSterne-1	None	None	Derived from Sterne Strain[d]
ΔTexas-1	None	None	Derived from Texas Strain[d]
ΔTexas-2	None	None	Derived from Texas Strain[d]

[a]Plasmid analysis by Green et al. (7), and B. Ivins (unpublished observations).

[b]United States Army Medical Research Institute of Infectious Diseases.

[c]Bovine isolate, Texas A&M University, College Station, Texas.

[d]Passaged daily for 10 days at 42.5°C, then twice purified from isolated colonies.

[e]American Type Culture Collection, Rockville, Maryland.

TABLE 2. Immunization of rats against toxin challenge[a]

Immunization		Serological Response[b]			Response to Toxin Challenge	
Strain	Plasmid Content	Anti-PA	Anti-LF	Anti-EF	Survival (%)	TTD[c]
Sterne	pXO1	6,400[d]	2016[d]	635[d]	100	----
Pasteur 4229	pXO2	<4	<4	<4	0	71.8 ± 2.2
ΔVollum 1B-1	pXO2	<4	<4	<4	0	67.3 ± 2.1
ΔVollum 1B-3	pXO2	<4	<4	<4	0	67.4 ± 1.5
ΔSterne-1	None	<4	<4	<4	0	68.8 ± 2.9
ΔV770-NPI-R-1	None	<4	<4	<4	0	67.2 ± 1.1
None	---	<4	<4	<4	0	76.0 ± 2.1

[a] Groups of male Fischer 344 rats, 200-300g, were immunized with 2×10^7 CFU of the indicated B. anthracis strain twice weekly for 4 weeks, given a final boost at 5 1/2 weeks, and challenged intravenously with 62 TU of anthrax toxin at 6 weeks.

[b] Reciprocal geometric mean ELISA titers of pre-challenge sera.

[c] Time to death (arithmetic mean minutes ± standard deviation).

[d] Statistically significant compared to unvaccinated controls: $P<0.05$.

TABLE 3. Immunization of guinea pigs against intramuscular spore challenge

Immunization		Serological Response[a]			Response to spore challenge[b]	
Strain	Plasmid Content	Anti-PA	Anti-LF	Anti-EF	Survival (%)	TTD[c]
Sterne[d]	pXO1	25,600[h]	4,526[h]	23	100	---
ΔVollum 1B-1[e]	pXO2	8	<4	5	0	2.55
Pasteur 4229[e]	pXO2	<4	<4	4	0	2.34
ΔSterne-1[e]	None	<4	<4	4	0	2.22
Texas[f]	pXO1	33,779[h]	6,400[h]	8,444[h]	100	---
ΔTexas-1[g]	None	<4	<4	<4	0	2.94
ΔTexas-2[g]	None	<4	<4	5	0	2.42
None	---	<4	<4	4	0	2.24

[a] Reciprocal geometric mean ELISA titers of pre-challenge sera.

[b] Two weeks following the final immunization, the guinea pigs received an i.m. challenge of approximately 30 LD_{50} of Vollum 1B spores.

[c] Harmonic mean time to death (days).

[d] Female Hartley guinea pigs, 275-325g, were given two injections, spaced two weeks apart, of 10^6 CFU Sterne-strain cells.

[e] Guinea pigs were given two injections, spaced two weeks apart, of 10^7 CFU of the indicated strain.

[f] Guinea pigs were given four biweekly injections of Texas-strain cells (first injection, 500 CFU, second, 1,500 CFU; third, 5,000 CFU; fourth, 10,000 CFU).

[g] Guinea pigs were given four biweekly injections of 10^8 CFU of the indicated strain.

[h] Statistically significant compared to unvaccinated controls: $P<0.05$.

TABLE 4. Immunization of guinea pigs against aerosol spore challenge[a]

Immunization		Serological Response[b]			Challenge	Response to Spore Challenge	
Immunogen	Plasmid Content	Anti-PA	Anti-LF	Anti-EF	Dose (LD_{50})	Survival (%)	TTD[c]
Sterne	pXO1	16,127[d]	8,064[d]	504[d]	26	100	--
MDPH vaccine	---	64,508[d]	3,311[d]	49[d]	26	71	20.47
ΔVollum 1B-3	pXO2	4	<4	<4	26	0	3.00
ΔTexas-1	None	4	6	4	26	0	3.53
ΔSterne-1	None	<4	4	<4	26	0	3.86
None	---	<4	<4	<4	26	0	3.11
Pasteur 4229	pXO2	<4	<4	<4	35	0	2.33
Pasteur 6602	pXO2	<4	<4	<4	35	0	2.80
None	---	<4	<4	<4	35	0	2.80

[a] Groups of female Hartley guinea pigs, 275-325g, were immunized either with MDPH (3 biweekly doses of 0.5 ml) or with the indicated *B. anthracis* strain (10^7 CFU in 4 biweekly doses). Two weeks after the final immunization the animals were given an aerosol challenge of either 26 or 35 LD_{50} of Vollum 1B spores.

[b] Geometric mean ELISA titers of pre-challenge sera.

[c] Harmonic mean time to death (days).

[d] Statistically significant compared to unvaccinated controls: $P<0.05$.